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Effect of high dietary copper on growth, antioxidant and lipid metabolism enzymes of juvenile larger yellow croaker *Larimichthys croceus*

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ABSTRACT

A study was carried out to test the responses of juvenile larger yellow croaker *Larimichthys croceus* to high Cu intake. Experimental diets were formulated containing three levels of Cu: low Cu (3.67 mg/kg), middle Cu (13.65 mg/kg) and high Cu (25.78 mg/kg), and each diet were fed to large yellow croaker in triplicate for 10 weeks. Final body weight, weight gain and feed intake were the lowest in high Cu group, but hepatosomatic index was the highest; Cu concentrations in the whole-body, muscle and liver of fish fed low Cu diet was the lowest; Liver superoxide dismutase, catalase and glutathione peroxidase activities in fish fed high Cu diet were lower than those in fish fed other diets; The higher content of liver thiobarbituric acid reactive substance content was found in high Cu group, followed by middle Cu group, and the lowest in low Cu group; Liver 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, malic enzyme, isocitrate dehydrogenase and fatty acid synthase activities were the lowest in high Cu group, but lipoprotein lipase activity was the highest. This study indicated that high copper intake reduced growth of juvenile larger yellow croaker, inhibited activities of antioxidant enzymes and lipid synthetases, and led to energy mobilization.

1. Introduction

Copper (Cu) is an essential trace element for fish and involved in numerous important biochemical reactions, such as hematopoiesis (Shao et al., 2010) and collagen synthesis (Lall, 2002). It is also important as a part of antioxidant enzymes (e.g. Cu–Zn SOD) and involved in oxidation–reduction reaction (Watanabe et al., 1997). However, the excessive accumulation of copper in the body causes toxicity (Lapointe et al., 2011). To date, it has become a challenge to keep the quantities of heavy metals in aquatic animal feed ingredients (e.g. fish meal, soybean meal and flour) under proposed maximum levels while heavy metal pollution is one of the major environmental issues (Clearwater et al., 2002). The previous studies found that exposure to Cu might affect fish growth performance (Chen et al., 2012), development (Carreau and Pyle, 2005) and reproduction (Pickering et al., 1977). Recently, Chen et al. (2013) found that Cu exposure could influence fish lipid deposition and lipid metabolism, the activities of lipogenic enzymes (e.g. fatty acid synthase) as well as mRNA levels of related genes decreased with increasing Cu concentrations, but activity and mRNA level of lipoprotein lipase gene increased. This study provides evidence that Cu exposure can disturb the normal processes of lipid metabolism of fish at both the enzymatic and molecular levels. At present, these studies mentioned above were conducted to determine the toxicity of Cu exposure, however, effect of high dietary Cu on fish growth, physiological and lipid metabolism remains unknown.

Large yellow croaker *Larimichthys croceus* is a commercially important marine species, highly preferred by consumers, and widely cultured in China. To our knowledge, no information is available concerning the effect of high dietary Cu on growth performance, antioxidant and lipid metabolism enzymes activities on juvenile larger yellow croaker. The results could shed light to enhance fish growth and health through dietary formulation.

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Table 1
Formulation and proximate composition of the experimental diets (% dry matter, DM).

	Low Cu	Middle Cu	High Cu
Ingredients			
Vitamin free casein	36.00	36.00	36.00
Gelatin	9.00	9.00	9.00
Dextrin	25.00	25.00	25.00
Fish oil	9.00	9.00	9.00
Lecithin	3.00	3.00	3.00
Vitamin premix[a]	2.00	2.00	2.00
Cu-free mineral mix[b]	1.00	1.00	1.00
Betaine	1.00	1.00	1.00
$Ca(H_2PO_4)_2$	1.00	1.00	1.00
Cellulose	12.95	12.95	12.95
Ethoxyquin	0.05	0.05	0.05
$CuSO_4{\cdot}5H_2O$ (mg/kg diet)	0.00	8.00	20.00
Proximate composition			
Moisture (%)	9.05	9.60	9.12
Crude protein (%)	45.91	46.01	45.58
Crude lipid (%)	9.71	9.83	10.01
Cu (mg/kg diet)	3.67	13.65	25.78

[a] Vitamin mix (mg or g/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine·HCl, 20 mg; vitamin B_{12}, 0.1 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1.2 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; α-tocopherol, 120 mg; ascorbic acid, 2000 mg; choline chloride, 2500 mg; microcrystalline cellulose, 14.1617 g.

[b] Cu-free mineral mix (mg or g/kg diet): $MgSO_4{\cdot}7H_2O$, 1200 mg; $FeSO_4{\cdot}H_2O$, 80 mg; $MnSO_4{\cdot}H_2O$, 44 mg, $ZnSO_4{\cdot}H_2O$, 50 mg; $CoCl_2{\cdot}6H_2O$, 50 mg; Na_2SeO_3, 20 mg; $Ca(IO_3)_2$, 60 mg; $Ca(H_2PO_4)_2{\cdot}H_2O$, 3000 mg; microcrystalline cellulose, 15.485 g.

2. Materials and methods

2.1. Experimental diets and animals

Three experimental diets, using casein and gelatin as protein sources and fish oil as the lipid source, were formulated to contain three graded levels of Cu [low Cu (3.67 mg/kg diet), middle Cu (13.65 mg/kg diet) and high Cu (25.78 mg/kg diet); Cao et al. (2014) reported that a dietary Cu requirement in juvenile large yellow croaker was 3.41 mg/kg diet]. Diets were processed into 3 mm diameter pellets, dried at room temperature to <10% moisture, ground and sieved to appropriate size before being stored at −20 °C. The formulation and proximate composition of each diet is presented in Table 1.

Juvenile large yellow croaker was obtained from a fish farm (Ningde, China). The fish were reared in floating sea cages (3.0 × 3.0 × 3.0 m) and were fed the commercial diet for 14 days. At the end of the acclimation, fish (5.27 ± 0.17 g, mean ± S.E.M.) were randomly stocked into 9 sea cages (1.5 × 1.5 × 2.0 m) with 60 fish each in triplicate. The fish were hand-fed experimental diets twice daily (05:00–06:00 and 17:00–18:00) to apparent satiation for 10 weeks. During the trial, the water temperature ranged from 21.0 °C to 25.0 °C, salinity 22–26‰ and dissolved oxygen concentration was about 6.8 ± 0.15 mg/L.

2.2. Experimental designs and sampling

At the termination of trial, all fish were starved for 24 h, and then were anesthetized with tricaine methanesulfonate (MS-222) at 120 mg/L for weighing, counting and measurement. Five fish from each cage were randomly sampled, minced, pooled and stored at −20 °C for the analysis of whole-body proximate composition and Cu concentration. Muscle (3 fish/cage) were sampled and stored at −20 °C for Cu concentration. Livers (3 fish/cage) were removed and individually weighed to determine the hepatosomatic index, and stored at −20 °C for Cu concentration, antioxidant and fatty acid metabolism enzymes activities analysis.

2.3. Biochemical composition analysis

All experimental diets and tissue samples were analyzed for proximate composition following the standard methods in triplicate (AOAC, 1995). Moisture was determined by oven drying at 105 °C to a constant weight. The samples used for dry matter were digested with nitric acid and incinerated in a muffle furnace at 550 °C overnight for ash determination. The protein was measured by the combustion method using an FP-528 nitrogen analyzer (Leco Corporation, St. Joseph, MI, USA). Lipid was determined by ether-extraction method using the Soxtec System HT (FOSS Tecator HT6, Hoganos, Sweden). Cu concentrations in the whole body and liver were determined by the inductively coupled plasma-atomic emission spectrophotometer (Vista-MPX, Varian).

2.4. Antioxidant enzyme activity and lipid peroxidation assays

The levels of enzyme activity and lipid peroxidation were measured with commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) in accordance with the instructions of the manufacturer. The assays are briefly described as follows:

The frozen liver were weighed and homogenized in ice-cold phosphate buffer (50 mM, pH 7.4). The homogenate was centrifuged at 2000 × *g* in a cooling centrifuge at 4 °C for 15 min and the supernatant was saved. Total superoxide dismutase (SOD) activity was determined following the methods of Beauchamp and Fridovich (1971). The ratio of auto-oxidation rates of the samples with or without homogenate was determined at 550 nm. One unit of SOD activity was calculated using the amount of superoxide dismutase required to inhibit the reduction of nitrobluete trazolium by 50%. Catalase (CAT) activity was determined by measuring the decrease in H_2O_2 concentration (Aebi, 1984). After 10 μL of homogenate was added to the reagent, the sample was incubated for 60 s at 37 °C. The absorbance of the samples was read at 405 nm. One unit of CAT activity was defined as the amount of CAT required to transform 1 μmol of H_2O_2 per min. Glutathione peroxidase (GPX) activity was measured following the methods of Flohé and Günzler (1984). After the addition of 1 mmol GSH (reduced glutathione) the NADPH-consumption rate was monitored at 412 nm. One unit of GPX activity was definedas the amount of GPX required to oxidize 1 μmol of NADPH per min. The terminal product formed in the decomposition of polyunsaturated fatty acids mediated by free radicals was quantified as thiobarbituric acid reactive substances (TBARS) according to the methods of Buege and Aust (1978). Soluble protein concentration of homogenates was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

2.5. Lipid metabolism enzyme activity assays

The frozen liver were weighed and homogenized in ice-cold buffer (0.02 M Tris–HCl, 0.25 M sucrose, 2 mM EDTA, 0.1 M sodium fluoride, 0.5 mM phenyl methyl sulphonyl fluoride, 0.01 M β-mercapo-ethanol, pH 7.4). The homogenatewas centrifuged at 20,000 × *g* in a cooling centrifuge at 4 °C for 30 min and the supernatant was saved. 6-Phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD) were determined by the method of Barroso et al. (1999), malic enzyme (ME) activity following Wise and Ball (1964), isocitrate dehydrogenase (ICDH) activity according to Bernt and Bergmeyer (1974), fatty acid synthase (FAS) activity according to the method of Chakrabarty and Leveille (1969). One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of substrate to product per min at 30 °C. Lipoprotein lipase (LPL) activity was measured according to the modified method of Ballart et al. (2003). One unit of LPL activity was defined as the amount of enzyme that catalyzed

Table 2
Growth performance and feed utilization of juvenile large yellow croaker fed diets supplemented with various levels of Cu for 10 weeks.

	Low Cu	Middle Cu	High Cu
FBW (g)	30.57 ± 0.71[b]	31.33 ± 0.90[b]	27.99 ± 0.82[a]
WG (g)	25.27 ± 0.55[b]	26.11 ± 1.09[b]	22.70 ± 0.88[a]
FI (g)	19.14 ± 0.20 [b]	19.73 ± 0.82 [b]	13.58 ± 1.80 [a]
FE	1.32 ± 0.02	1.33 ± 0.02	1.33 ± 0.02
HSI (%)	1.41 ± 0.04[a]	1.40 ± 0.08[a]	1.62 ± 0.05[b]
CF	1.75 ± 0.04	1.75 ± 0.04	1.73 ± 0.07
Survival (%)	80.00 ± 5.00	76.11 ± 5.09	77.22 ± 6.93

Data are means of triplicate. Means in the same line sharing a same superscript letter are not significantly different determined by Duncan's test ($P > 0.05$). FBW (g): final body weight; FI (g): feed intake; weight gain (WG, g) = final individual weight (g) – initial individual weight (g); Feed efficiency (FE) = wet weight gain (g)/dry diet fed (g); hepatosomatic index (HSI, %) = 100 × liver mass (g)/body mass (g); condition factor (CF) = 100 × weight gain (g)/body length (cm)^3; survival (%) = 100 × (finial number of fish)/(initial number of fish).

Table 3
The whole-body compositions of juvenile large yellow croaker fed diets supplemented with various levels of Cu for 10 weeks.

	Low Cu	Middle Cu	High Cu
Moisture (%)	73.34 ± 0.28	73.36 ± 0.25	73.25 ± 0.25
Protein (% DM)	16.78 ± 0.27	16.57 ± 0.38	16.39 ± 0.19
Lipid (% DM)	8.05 ± 0.10	8.33 ± 0.31	8.27 ± 0.22
Ash (% DM)	3.52 ± 0.03	3.55 ± 0.03	3.51 ± 0.04

Table 4
The whole-body, muscle and liver Cu concentrations of juvenile large yellow croaker fed diets supplemented with various levels of Cu for 10 weeks (μg/g).

	Low Cu	Middle Cu	High Cu
Whole-body Cu	8.41 ± 0.15[a]	10.63 ± 0.34[b]	10.80 ± 0.24[b]
Muscle Cu	7.53 ± 0.40 [a]	8.78 ± 0.88 [b]	9.29 ± 0.29 [b]
Liver Cu	9.84 ± 0.28[a]	12.92 ± 0.13[b]	12.99 ± 0.12[b]

Data are means of triplicate. Means in the same line sharing a same superscript letter are not significantly different determined by Duncan's test ($P > 0.05$).

the release of 1 μmol of free fatty acid per hour per mg of soluble protein at 37 °C. Soluble protein concentration of homogenates was determined by the method of Bradford (1976).

2.6. Statistical analyses

All variables were assessed using one-way ANOVA. If there was a significant F-test, subsequent comparisons of treatment means were performed using the Duncan's Multiple Range test. The significance level was set at $P < 0.05$. All analyses were performed using SPSS 18.0.0 (Chicago, USA) for windows.

3. Results

3.1. Growth performance and body composition

Survival rate (>76.11%) was not different among all experimental groups ($P > 0.05$, Table 2). Fish fed with high Cu diet had the lowest final body weight, weight gain and feed intake ($P < 0.05$), no significant differences were found between low and middle Cu diets ($P > 0.05$). On the contrary, hepatosomatic index of fish fed high Cu diet were the highest ($P < 0.05$). However, the dietary Cu level did not significantly affect feed efficiency and condition factor ($P > 0.05$). Moisture, protein, lipid and ash contents in the whole body did not show any significant differences among the dietary treatments ($P > 0.05$, Table 3). Fish fed with low Cu diet had the lowest whole-body, muscle and liver Cu concentrations among all treatments ($P < 0.05$), no significant differences were found between middle and high Cu diets ($P > 0.05$, Table 4).

Table 5
Antioxidant enzyme activity and lipid peroxidation of juvenile large yellow croaker fed diets supplemented with various levels of Cu for 10 weeks.

	Low Cu	Middle Cu	High Cu
SOD (U/mgprot)	108.11 ± 5.65[b]	103.44 ± 4.71[b]	90.61 ± 0.41[a]
CAT (U/mgprot)	38.82 ± 0.29[b]	38.91 ± 0.35[b]	31.16 ± 0.88[a]
GPX (U/mgprot)	383.20 ± 2.01[b]	381.77 ± 8.88[b]	307.42 ± 5.58[a]
TBARS (nmol/mgprot)	53.71 ± 1.93[a]	73.17 ± 1.78[b]	81.42 ± 0.80[c]

Data are means of triplicate. Means in the same line sharing a same superscript letter are not significantly different determined by Duncan's test ($P > 0.05$). SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; TBARS: thiobarbituric acid reactive substances.

Table 6
Fatty acid metabolism enzyme activity of juvenile large yellow croaker fed diets supplemented with various levels of Cu for 10 weeks (mIU/mg protein).

	Low Cu	Middle Cu	High Cu
6PGDH	194.74 ± 4.57[b]	191.47 ± 3.65[b]	125.87 ± 4.62[a]
G6PDH	253.45 ± 2.12[c]	225.39 ± 3.80[b]	207.61 ± 5.24[a]
ME	45.01 ± 0.12[b]	44.58 ± 0.65[b]	25.92 ± 0.13[a]
IDH	176.70 ± 0.40[c]	161.73 ± 0.63[b]	154.77 ± 0.39[a]
FAS	50.60 ± 0.40[c]	25.85 ± 0.36[b]	25.14 ± 0.17[a]
LPL	365.34 ± 0.89[a]	447.74 ± 5.60[b]	507.52 ± 5.03[c]

Data are means of triplicate. Means in the same line sharing a same superscript letter are not significantly different determined by Duncan's test ($P > 0.05$). 6PGDH: 6-phosphogluconate dehydrogenase; G6PDH: glucose-6-phosphate dehydrogenase; ME: malic enzyme; IDH: isocitrate dehydrogenase; FAS: fatty acid synthase; LPL: lipoprotein lipase.

3.2. Antioxidant enzyme activity

Serum superoxide dismutase, catalase and glutathione peroxidase activities of fish fed high Cu diet were significantly lower than low and middle Cu diets ($P < 0.05$), but existed no significant differences between low and middle Cu diets ($P > 0.05$, Table 5). The higher content of liver thiobarbituric acid reactive substance content was found in high Cu group, followed by middle Cu group, and the lowest in low Cu group ($P < 0.05$).

3.3. Fatty acid metabolism enzyme activity

Fish fed with high Cu diet had the lowest activities of 6-phosphogluconate dehydrogenase and malic enzyme ($P < 0.05$), no significant differences were found between low and middle Cu diets ($P > 0.05$, Table 6). The highest glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and fatty acid synthase activities were found in fish fed low Cu diet, coming with middle Cu diet, the lowest is high Cu diet ($P < 0.05$). On the contrary, the highest lipoprotein lipase activity was found in fish fed high Cu diet ($P < 0.05$).

4. Discussing

In the present study, a higher level of Cu intake was employed, survival rate showed no significant difference among the treatments, indicating that larger yellow croaker was not sensitive to high Cu intake, which is in agreement with the finding in grouper *Epinephelus malabaricus* (Lin et al., 2008) and yellow catfish *Pelteobagrus fulvidraco* (Chen et al., 2015). Cao et al. (2014) reported that the optimal dietary Cu concentration in growing large yellow croaker is 3.41 mg/kg diet. In this study, fish weight gain was reduced when Cu intake exceeded the optimum requirement 6 times (25.78 mg/kg). Growth reduction due to high Cu intake has been reported in several fish species, such as tilapia *Oreochromis niloticus* × *Oreochromi saureus* (Shiau and Ning, 2003), grouper (Lin et al., 2010), yellow catfish (Tan et al., 2011) and grass carp *Ctenopharyngodon idellus* (Tang et al., 2013). The reduction in

growth performance might be attributable to increased metabolic expenditure for detoxification and maintenance of homeostasis (Campbell et al., 2002). Chen et al. (2013) reported high Cu exposure significantly reduced fish weight gain, condition factor, viscerosomatic index, hepatosomatic index and visceral adipose index, because of higher Cu exposure reduced feed intake. In this study, feed intake of fish fed high Cu diet were significantly the lowest, but increased hepatosomatic index of fish after high Cu intake might be attributable to liver swelling. A previous study has found that dietary copper was transported from intestinal mucosa into blood by combined with superoxide dismutase or metallothionein, but high levels of copper may lead to over-consumption of carrier albumin, and caused free fat metabolic disorders and liver lesions (Qian et al., 2003).

The whole-body Cu concentration of experimental animal was positively correlated to the food. Previous studies found, after being fed with increasing levels of dietary Cu for about 1 week, Cu would accumulate to the higher concentration in intestine and liver of fish (Clearwater et al., 2000; Kamunde et al., 2001). In the present study, the concentration of Cu in the whole body increased with dietary Cu levels. It is indicated that large yellow croaker could accumulate excess Cu in tissues. Berntssen et al. (1999) reported that the whole-body Cu concentration in Atlantic salmon *Salmo salar* increased with the increasing dietary copper levels. Cao et al. (2014) also found the whole-body Cu concentration in large yellow croaker increased with the increasing dietary copper levels. In addition, the liver and muscle Cu concentrations have the similar trends with the whole-body Cu concentration in the present study.

The cellular damage would be enhanced by lipid peroxidation due to reactive oxygen species (ROS) overproduction (Ito et al., 1999). The aerobic condition of fish makes them prone to the generation of ROS as a result of an exacerbated oxidative metabolism, resulting in lipid peroxidation (Takagi et al., 2008). Aldehyde (e.g. malondialdehyde) is a product of lipid peroxidation, through cross linking with the nucleophilic groups of proteins, nucleic acids and amino phospholipids, accumulation of aldehyde leads to cell toxicity, accelerating the damage of cells and tissues (Buege and Aust, 1978). In this study, fish fed the experimental diet containing 25.78 mg/kg Cu had significantly higher thiobarbituric acid reactive substance concentration compared with fish fed the diets containing 3.67 and 13.65 mg/kg Cu. Under normal conditions, the antioxidant defenses of fish prevent the uncontrolled generation of ROS through antioxidant enzymes (Trenzado et al., 2009). However, in this study, lower antioxidant enzyme activity occurs when fish are fed high Cu diet. Our result indicated that excessive Cu is detrimental to anti-oxidative capability, which is in agreement with the finding in Goldfish *Carassius auratus* (Shao et al., 2010) and large yellow croaker (Cao et al., 2014)

Chen et al. (2013) found that Cu exposure significantly reduced 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase and fatty acid synthase activities of yellow catfish, which were attributable to the reduction in the mRNA expression of the genes encoding them, indicating that these enzymes were regulated by Cu mainly at the transcriptional level. In this study, the highest glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and fatty acid synthase activities decreased with the increasing dietary copper levels. In addition, Hung et al. (1997) suggested that feed intakes will influence metabolic enzymatic activities. In the present study, feed intake of fish fed high Cu diet was lower than that of fish fed middle and low Cu diets. Here, we should bear in mind that the reduction of lipogenic enzyme activities might be attributable to high Cu intake. Lipoprotein lipase was considered as a key rate-limiting enzyme in the provision of tissue fatty acids, and it determined how dietary lipids were partitioned toward storage or utilization (Saera-Vila et al., 2005). In the present study, lipoprotein lipase activity increased with the increasing dietary copper levels, which might indicate an increase in import of lipids from liver to nearby tissues for energy mobilization.

The results of the present experiments indicated that excessive Cu reduced growth performance of juvenile larger yellow croaker; Cu could accumulate to the higher concentration in whole-body and liver of fish; High Cu intake inhibited activities of antioxidant enzyme and lipid synthetase, and led to energy mobilization.

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